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14. ABSTRACT
The overall goal of this project is to determine whether electrical stimulation of the spinal cord can reduce respiratory dysfunctions occurring after mid-to-high cervical spinal cord injuries (cSCI). Our primary emphasis is on closed-loop, intraspinal microstimulation (ISMS) of the phrenic circuit using physiologically-appropriate, endogenous respiratory signals to trigger activation of the phrenic motoneuron (PhMN) pool following either cSCIs above or at the level of the phrenic nucleus at spinal levels C3-C5/6 in adult rats. A major accomplishment of our studies thus far is demonstration of proof-of-concept for our closed-loop strategy before and after a spinal hemisection at C2 which results in immediate paralysis of the ipsilateral hemidiaphragm. Our studies have established that ISMS at the level of the PhMN can effectively activate diaphragm motor units following high cSCI even beyond when stimulation ended. Per comments from our proposal's initial review, we also began looking at the efficacy of high frequency (open-loop) spinal (epidural) stimulation. Our data indicate this approach is not effective at selectively activating inspiratory diaphragm (phrenic) motor units. These and other areas of progress lend considerable initial strength to the potential therapeutic value of closed-loop ISMS activation of respiratory circuits caudal to SCI.

15. SUBJECT TERMS
Respiration and cervical spinal cord injury, intraspinal microstimulation, epidural stimulation, Neurochip

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1. INTRODUCTION:

Compromised breathing (i.e., respiratory insufficiency) is one of the more devastating and potentially life-threatening consequences of spinal cord injury (SCI) at mid- to upper-neck (i.e., cervical) levels due to disrupted connections between respiratory centers in the brainstem and circuits controlling ventilatory muscles in the spinal cord. Lesions at or above C4 often result in severely, if not completely, reduced diaphragm function. This muscle, which is often considered the primary muscle of inhalation, receives its motor innervation from the phrenic nucleus primarily located at spinal C3-C5. Depending upon the severity and symmetry of the injury, descending inspiratory drive from the brainstem to the phrenic nucleus can result in uni- or bilateral paralysis of the diaphragm. Severe impairment of diaphragm function requires individuals to receive some form of assisted ventilatory support (e.g., mechanical, phrenic nerve, or diaphragm pacing). Even if some individuals are weaned, patterns of breathing rarely approximate normal, and pulmonary infection-related hospitalizations and deaths remain common. A need thus exists for interventions that can lead to a greater and more normal restoration of diaphragm and other respiratory muscle functions. Studies being conducted under this Department of Defense award are based upon increasing evidence showing that electrical stimulation of the injured spinal cord can elicit some degree of motor function (e.g., standing, limb movements) in animals and humans even with motor complete SCIs. The most common strategy involves epidural stimulation in which an electrical current is delivered to an electrode array placed on the surface of the spinal cord. Other studies are now emerging, however, which show that insertion of tiny microwires directly into spinal cord tissue (intraspinal microstimulation, ISMS) may produce more natural motor improvements. Epidural high frequency spinal stimulation has been shown to offer potential for improving aspects of respiratory function, but it is still dependent upon delivery of programmed signals (i.e., open-loop). We are now proposing to explore the capacity of ISMS to improve diaphragm function when delivered to phrenic circuitry. An innovative feature of our approach is a custom-designed circuit that will be used to capture signals from unaffected respiratory muscles. Those signals will then be used to stimulate the phrenic circuit in a natural and physiologically appropriate fashion under normal and more demanding ventilatory conditions. The intriguing possibility also exists this type of closed-loop strategy may lead to persistent recovery by directing functional and anatomical circuit remodeling and long-term effects no longer dependent upon stimulation. We will also determine differences in outcomes via epidural vs. ISMS. Overall, we are proposing the first investigation of a promising approach for stimulating respiratory circuits in two SCI models involving lesions above or within the phrenic circuit. Success of this project can lead to a significant shift in current approaches for managing respiratory dysfunction following cervical SCI. Knowledge obtained from this study also may have broader implications related to the use of ISMS for treating other aspects of SCI alone or in combination with other interventions.

2. KEYWORDS:

respiration
cervical spinal cord injury
intraspinal microstimulation
epidural stimulation
Neurochip
phrenic nucleus
phrenic motoneurons
phrenic neurograms
hypoglossal nerve
hypercapnia
hypoxia
neuroplasticity
C2 hemisection
mid-cervical contusion injury
interneurons
closed-loop stimulation
open-loop stimulation

3. INTRODUCTION:

Preface: While this first Annual Progress Report covers the period when the award was first issued (09-Sept-2014) to the present, funds were not released until ACURO approval which took longer than anticipated. Our initially approved IACUC was out of synchrony with the revised SOW which incorporated reviewer critiques. In addition, there was some miscommunication regarding the initial ACURO submission of 1/7/2015 which was based on the approved IACUC which covered the majority of studies for Year -01. A complete package of ACURO review was submitted 3/10/2015 after approval of a modified IACUC covering the entire project. ACURO approval was received 4/02/2015. The University of Washington subcontract also was kept on hold due to ACURO issues beyond investigators' control. That ACURO was approved on 05/14/2015. We are pleased to report, therefore, that the extensive progress we have been able to make reflects work performed in accordance with our approved SOW over the course of the last 5-6 months. Some of aspects of our study thus far also begin to extend into goals set forth for Year-02 of this award.

Major Goals of the Project: The following is a listing of major goals set forth in our revised and approved SOW which also identifies with comments raised during the initial review of this application. Table 1 below summarizes the scheduling of Major Tasks for performing experiments during Year -01, but not necessarily completing them during the first 12 months. Major Tasks 1-3 were to be conducted primarily at the University of Florida during Year-01. Major Tasks 4 and 5 were scheduled to be conducted at the University of Washington (per subcontract via this award which did not arrive until July 2015) during Year-01, as well as initiation of Major Task 7 during the second half of Year -01. Major Task 6, Subtask 1 was scheduled for the end of Year -01. The remaining Major Tasks are scheduled for Year -02. Due to delays in ACURO approval and resulting delays in sub-contract initiation, many of the year 1 tasks below are still in progress especially at the University of Washington.

TABLE 1

Major Task	Schedule (Months)	Work Site
Major Tasks 1-3	~2-12	University of Florida
Major Tasks 4, 5	~2-12	University of Washington
Major Task 6, Subtask 1	11	Univ. Florida and Univ. Washington
Major Task 7	~2-12	University of Washington
Major Task 6, Subtask 2-Major Task 11	13-24	Univ. Florida and Univ. Washington

University of Florida

Major Task 1: To determine chronic changes in neuronal discharge patterns within the phrenic circuit region following a C3/4 lateralized contusion injury.

This task was originally scheduled for Year -02 as a prelude to our proposed studies of spinal stimulation after a lateralized contusion injury at the level of the phrenic motoneuron pool. The main purpose of this study is to determine changes in the relative frequency of respiratory interneurons following C3/4 contusion injuries and C2 hemisection lesions which became part of the revised SOW. The results also will yield a baseline for determining whether effective spinal stimulation will alter the neurophysiological "signature" of the phrenic circuit. With other changes that had to be made in our SOW and with consideration of the labor-intensive nature of these experiments, the task was moved to Yea- – 01 to ensure completion before the contusion injury experiments were pursued.

Table 2

Experiment	Multi-electrode Array Recordings	Analyzed
Naïve	12	7
C2 Hemi-section	9	5
Lateralized C3/C4 Contusion	9	0

Using our established models of cervical spinal cord injury (Lane et al., 2008; Lane et al., 2012), we studied cervical neuron

discharge patterns in our anesthetized rat model (Sandhu et al., 2015) by recording with microelectrode array assemblies. As by Table 2, we have completed data collection for this aim, and analyses of these data are on-going. Dr. David Baekey, one of the co-investigators on this project, played a significant role in overseeing this work. We are scheduled to present these data at the Spring 2016 Experimental Biology Meeting in San Diego, CA, and thus fully anticipate that analysis will be completed over the next six months. We anticipate peer-reviewed to be forthcoming thereafter. Our key findings are summarized below and followed by representative examples of the data.

- The mid-cervical spinal cord contains a complex interneuronal network that displays respiratory-related neural discharge.
- The vast majority of spinal "respiratory interneurons" (Lane, 2011) (i.e., interneurons which burst in phase with the respiratory cycle) in the mid-cervical cord do not appear to be directly synaptically coupled (i.e., "antecedent") to phrenic motoneurons.
- The majority of mid-cervical spinal neurons respond to a respiratory challenge (hypoxia) by altering discharge rates, and repeated bouts of hypoxia evokes a persistent change in the bursting of most mid-cervical neurons.
- In naïve (i.e., spinal intact) rats, the majority of recorded neurons (66%) had a tonic discharge pattern, while a smaller proportion (34%) bursted in a phasic (inspiratory or expiratory) pattern. Spike triggered averaging revealed that the bulk of the tonic and phasic firing neurons were interneurons (Tonic IN: 93%; Phasic IN: 59%) compared to motor neurons (Tonic MN: 6%; Phasic MN: 41%).
- Following lateral C2 spinal hemisection injury, a greater number of neurons below the injury have a tonic firing pattern (85%) relative to that observed in naïve rats (66%), whereas contralateral to injury there was an increase in the number of phasic firing neurons (68%) compared to naïve rats (34%).
- As noted above, all multi-electrode array recordings following contusion are complete and analyses will be completed in the coming months.

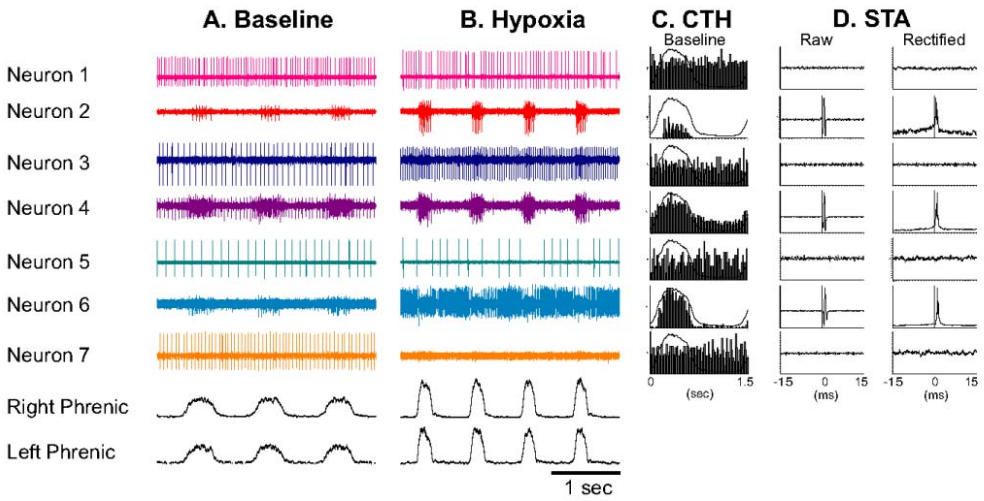

Figure 1

Figure 1 is an example of the activity exhibited by simultaneously recorded mid-cervical spinal neurons. Example recordings from 7 cervical (C4) spinal neurons and integrated phrenic nerve output ipsilateral (left) and contralateral (right) to multi-electrode recordings during A) baseline and B) hypoxia (FiO_2: 11%) depicting a complex network that responds to hypoxia. C). Cycle triggered histograms (CTH) of each neuron during 50 consecutive breaths at baseline overlaid with the average integrated phrenic waveform. D). Spike triggered averages (STA) of the raw and rectified ipsilateral (left) phrenic nerve. A positive feature in both the raw and rectified phrenic nerve with a short-latency (<1 msec)

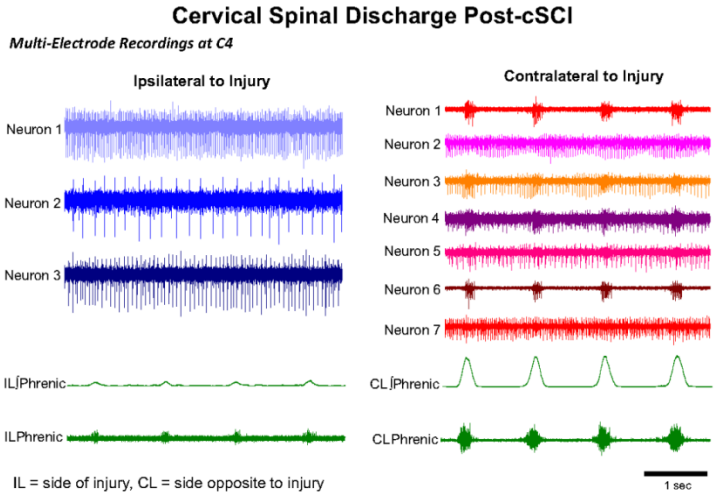

Figure 2

was observed for 3 neurons (neurons 2, 4 and 6) providing evidence that the recorded neurons were phrenic motoneurons. Remaining neurons (neurons 1, 3, 5 and 7) without a positive feature were classified as interneurons. These data were obtained from a spinal-intact rat.

Figure 2 demonstrates mid-cervical spinal discharge following cervical spinal cord injury. Representative bilateral multi-electrode recording at spinal segment C4, and raw and integrated phrenic nerve activity recorded ipsilateral and contralateral to lateral cervical hemisection injury. These data demonstrate that the majority of neurons below injury burst in a tonic firing pattern whereas most of recorded neurons contralateral to injury burst in phase with breathing. Data were recorded 12 weeks post-injury. Pending further analyses, these initial data suggest that complete interruption of bulbospinal inspiratory drive, which is preferentially directly to phrenic motoneurons, may have consequences elsewhere at the level of the phrenic circuit. Whether this reflects a change in the physiological state of the phrenic circuit itself and how that might be affected by spinal stimulation will become of more interest as this project moves forward.

Over the last several weeks, we have developed a method to label the location of the recording electrodes in the cervical spinal cord. Figure 3 depicts: **A)** A schematic of the multi-electrode recording array caudal to C3/C4 lateralized hemi-contusion. **B)** Raw discharge from an intraspinal neuron recorded from electrode 8 and integrated phrenic nerve activity. **C)** Bright field image (5X) of a C4 spinal section counter stained with cresyl violet depicting positive silver labeling of electrode 8. **D)** Higher magnification (20x) of silver labeling (inset).

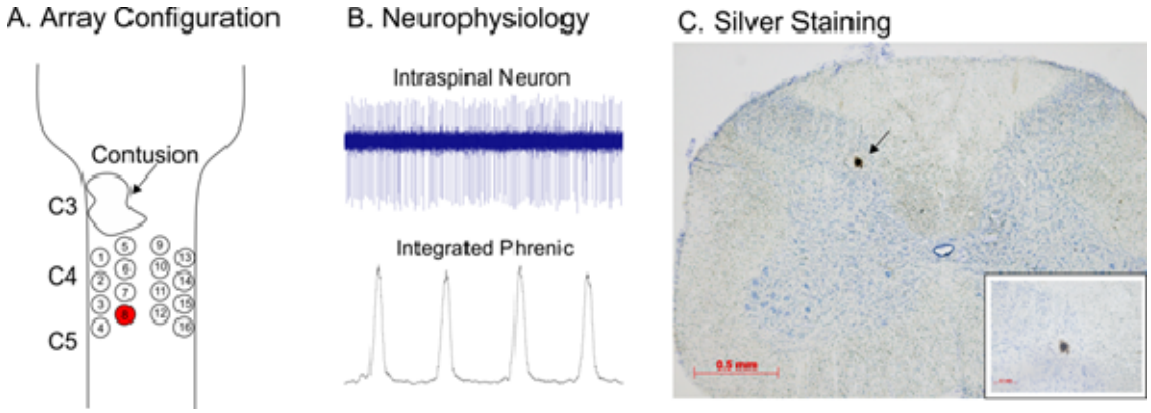

Figure 3. Mid-cervical spinal neuron activity and histology 5 weeks post-lateralized C3/C4 contusion (200kD).

Major Task 2: *Terminal electrophysiological comparison of epidural and ISMS stimulation on activation of the phrenic motor circuit after chronic C2 hemisection (C2Hx; one month post-SCI).*

Intraspinal Microstimulation

We completed an initial proof-of-concept study to determine if ISMS in the cervical spinal cord could activate diaphragm motor units, and whether the ability to activate respiratory activity persisted following a cervical SCI. We hypothesized that a real-time feedback, or a “closed-loop”, cervical ISMS would recruit diaphragm motor units that had been deprived of descending drive from the brainstem from an acute cervical spinal cord injury in an endogenous rhythmic manner.

To address these hypotheses we investigated the changes in respiratory motor output electrophysiologically in a rodent model from ISMS targeting phrenic motoneurons in the cervical spinal cord. Closed-loop ISMS was triggered by the endogenous respiratory rhythm off of tongue muscle activity to be delivered during neural inspiration. During eupnea, only a portion of phrenic motoneurons are recruited during inspiratory efforts and there remains a reserve that can be activated by stimulation (Mantilla and Sieck, 2011). To confirm activation of diaphragm motor units by stimulation, pulses were delivered during expiration, a period that the phrenic motoneurons are inhibited (Berger, 1979). The effect of closed-loop ISMS on activating diaphragm motor units also was examined after lesioning one side of the spinal cord (lateral C2 hemisection). This model abolishes spontaneous hemidiaphragm activity

(Goshgarian, 2003). The experiments were successful, and as shown in Figures 4-6, we have established that ISMS of the mid-cervical spinal cord can effectively activate diaphragm motor units. Furthermore, the method can activate diaphragm motor units following cervical spinal cord injury (Figs. 4-5). We also found that following ISMS, function of the paralyzed diaphragm persisted even *after the stimulation has been stopped* (Fig. 6). This finding is an independent corroboration of results reported by our Seattle colleague in a study of ISMS effects on forelimb function after cervical contusion injury (Kasten et al., 2013).

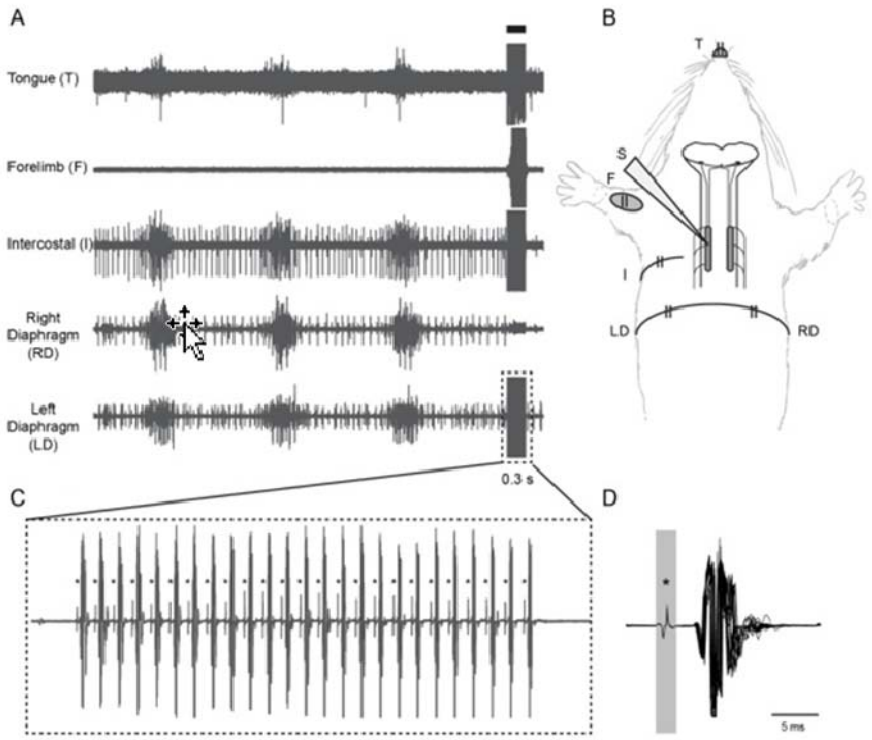

Figure 4. Raw EMG activities recorded in the tongue, forelimb, intercostal, right and left diaphragm.

Figure 4 illustrates representative traces of raw EMG activities recorded in the tongue, forelimb, intercostal, right and left diaphragm (6 s window, panel A). The highlighted area in panel A shows a period of ISMS (100 Hz; 200 uA). A schematic of the experimental preparation showing the location of muscles recorded and location of stimulating electrode at C4 level in the left lateral spinal cord targeting the phrenic motoneuron pool is provided in panel B. Panel C an expanded trace of the left diaphragm raw EMG (0.3 s window) during ISMS stimulation shows activation following each stimulus pulse. An "overlay" of each ISMS-evoked action potential is shown in panel D. Stimulus artifact marked with asterisks. The major response using hypoglossal triggering is in the diaphragm and forelimb. This is not surprising as we had anticipated this possibility given the highly overlapped nature of the motoneuron pools and pilot data provided in our proposal. While that issue will ultimately need to be resolved or used to further benefit, the main goal of our project is focused on the phrenic nucleus and diaphragm. The initial data fully support our hypothesis that ISMS, using naturally-derived stimuli, has the potential for re-activating a previously silenced phrenic circuit.

Shown in Figure 5 are 3 spontaneous breaths followed by 3 breaths during closed-loop ISMS before and immediately following a C2 hemisection (C2Hx). The effect of the left lateral hemisection injury is schematically represented to show the disconnection of spinal pathways connecting the brainstem and phrenic motoneuron pool in panel C. The lesion causes a loss of spontaneous inspiratory bursting activity of the left diaphragm EMG. Stimulus triggered averages (panel D) for the right and left diaphragm show that similar activation was accomplished by stimulation with

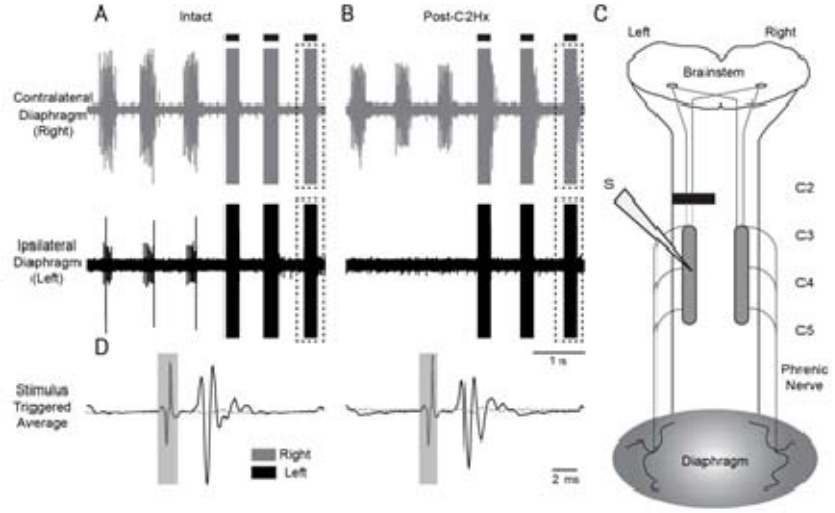

Figure 5. Raw EMG traces of the left and right diaphragm in the intact spinal cord (panel A) and post-spinal cord injury (panel B).

spinal cord intact (panel D, left) and following spinal cord injury (panel D, right). Stimulation after injury was applied 10-min after all spontaneous activity had ceased on the left diaphragm EMG.

As shown in Figure 5, C2Hx causes paralysis of the ipsilateral diaphragm (note lack of EMG, Fig. 6). However, closed-loop ISMS triggered from the hypoglossal motor output (gray arrows, Fig. 6) evoked robust diaphragm activity during the period of stimulation. In addition, the ISMS was able to elicit a partial recovery of spontaneous diaphragm activity immediately post-stimulation (far right panel).

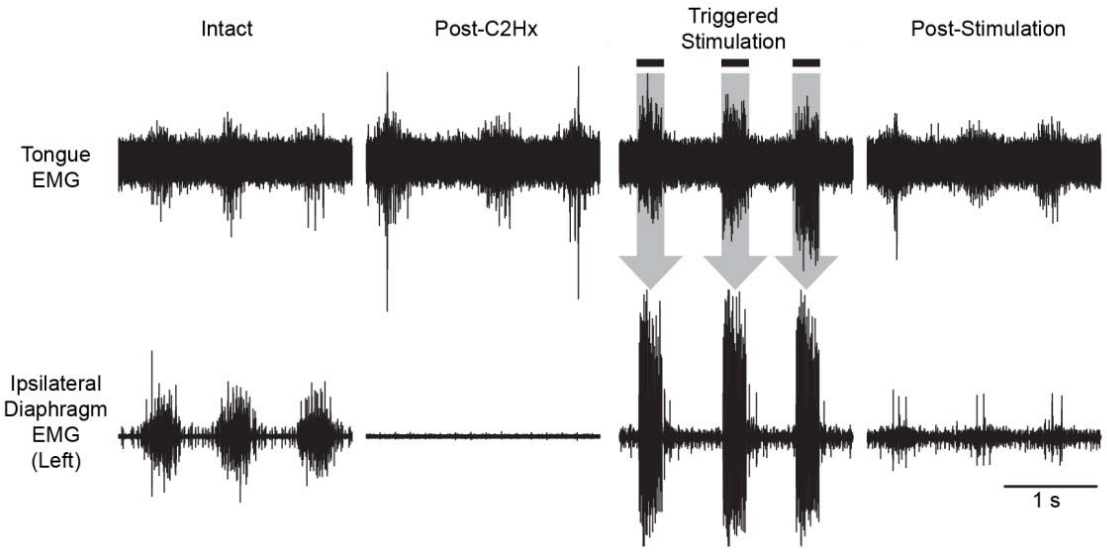

Figure 6. Representative raw EMG activity from the tongue and ipsilateral diaphragm (left) in the intact spinal cord, post-spinal cord injury (C2Hx or hemilesion), during closed-loop ISMS (100 Hz; 200 uA), and immediately post-stimulation.

Epidural Stimulation

The pioneering work of DiMarco's group (DiMarco and Kowalski, 2010; 2011) establishes high frequency epidural stimulation as a highly effective "respiratory neuroprosthesis" after an acute, complete C1 spinal transection in animals. However, the far more common clinical condition is incomplete cervical SCI in which partial control of the diaphragm is maintained (Lane et al., 2008). Recent work from UCLA and Louisville has received considerable media attention by showing that epidural stimulation can produce both somatic motor and autonomic benefits after incomplete SCI in humans (Harkema et al., 2011; Rejc et al., 2015). Indeed, there are now multiple examples of partial restoration of voluntary motor control during epidural stimulation in persons with SCI. The underlying mechanisms are not clear, but one hypothesis is that epidural stimulation can raise the overall "excitability" of the local spinal network, thereby allowing the small amount of preserved synaptic input from rostral centers to activate spinal motor circuits. Together, the animal and human data (Harkema et al., 2011; Rejc et al., 2015) raise an **important question**: rather than serving as an "open loop" respiratory neuroprosthesis that provides the only input to respiratory motoneurons, *can epidural stimulation modulate the spinal network in a way that enables the endogenous (medullary) respiratory control circuits to more effectively control the respiratory muscles after incomplete SCI*? Thus, we studied epidural stimulation and respiratory function after chronic incomplete cervical SCI.

We used a rat SCI model (C2 hemisection, C2Hx) to test the hypothesis that epidural stimulation can modulate respiratory motor output after chronic incomplete cervical SCI. Since rats with chronic C2Hx display a small degree of recovered spontaneous inspiratory motor activity in the phrenic motor pool ipsilateral to the lesion attributed to the crossed phrenic phenomenon (Goshgarian, 2003), C2Hx provides a model to examine the impact of epidural stimulation on endogenous respiratory activity. Thus, neurophysiology experiments were conducted after subacute (2 wks) and chronic C2Hx lesion (12 wks) with HF-ES delivered at the level of (e.g. at C4) or distal to the phrenic motor pool (e.g. at T2). Phrenic motor output was examined before and after a brief period (60 s) of low (100 uA), medium (500 uA), and high (1000 uA) intensity stimulation to determine whether spinal HF-ES could induce short-term plasticity of phasic inspiratory phrenic discharge.

The data indicate that epidural stimulation with the method of DiMarco *et al.*(Dimarco and Kowalski, 2013; Kowalski et al., 2013) is not effective at selectively activating inspiratory diaphragm (phrenic) motor units. Rather, when we tried epidural stimulation after chronic incomplete cervical SCI, we observed robust increases in tonic phrenic/diaphragm activity (*i.e.*, activity across the entire respiratory cycle), with very little change in the phasic (inspiratory) phrenic burst.

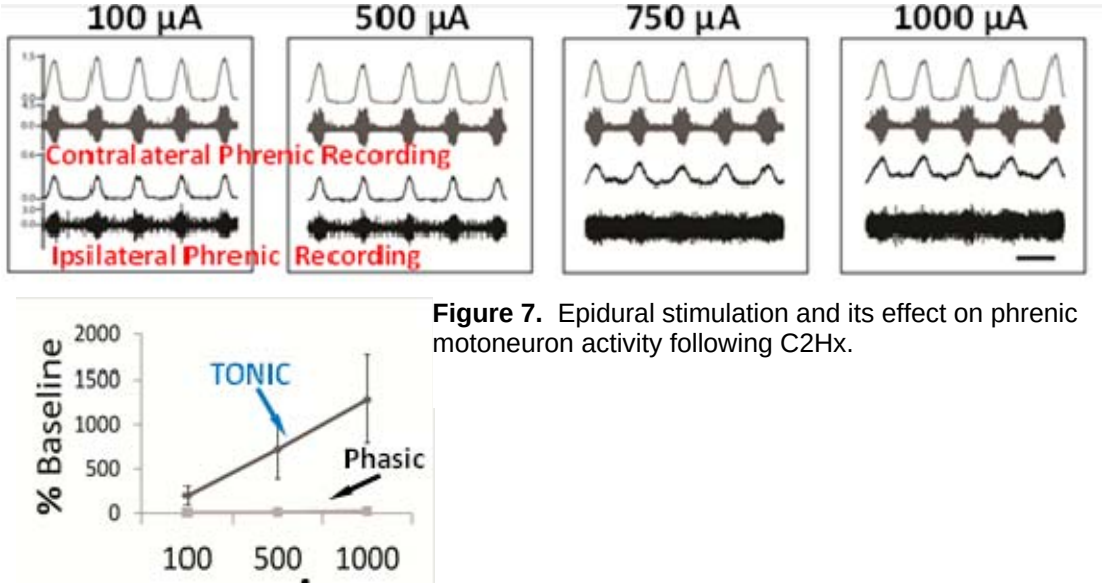

Figure 7. Epidural stimulation and its effect on phrenic motoneuron activity following C2Hx.

High frequency epidural stimulation of the ventrolateral cervical (C3-4) spinal cord was performed rats with C2Hx injury, which produced tonic discharge in the ipsilateral phrenic nerve (bottom traces, tonic eventually "swamps" the phasic signal, Fig. 7). There was little impact on contralateral phrenic output – thus the effects of stimulation were localized. The mean ipsilateral phrenic response is shown in the lower panel of Fig. 7. Quantitatively similar data were obtained at sub-acute (2 wks) and chronic (8-12 wks) C2Hx injury and with both cervical and thoracic epidural stimulation.

Major Task 3: Analysis of Tissue Responses to Microwire Implantation

These experiments, which were incorporated into the revised SOW per comments made by the review panel, have begun to examine the nature and timing of pathological responses to microwire insertion, which others have reported in different models of ISMS CNS stimulation. The experiment involves insertion of microwires (no electrical stimulation) into the gray matter regions of the uninjured C3-5 spinal cord followed by terminal perfusion of animals with fixative at 3, 7, and 14 days post-wire insertion. Initial histological examination indicated an early presence of cells surrounding the wires. The immediate conclusion is that the insertion procedure introduced cells from meningeal areas that infiltrated the breached spinal cord. The temporal dynamics of this response is currently being investigated.

Major Task 5: Conduct Phase I of closed-loop intraspinal stimulation of phrenic motor neurons (PhMNs) after C2Hx.

We made considerable progress towards this goal. Part of the progress was reviewed above (Figs. 4-6) as a component of major task 2. However, we also conducted initial studies using the NeuroChip as described in the original proposal. Dr. Mortiz (Seattle) sent his lead technician, Mr. Michael Sunshine, to Gainesville to begin the collaborative process. Sunshine brought the Neurochip, and began the process of training the Gainesville team to use it. We were successful with our initial efforts, as outlined in Fig. 8. Stimulations were delivered using a custom-made electrode array implanted in the subdural space on the left side of the spinal cord at C4. Flush-cut platinum microwires were inserted into the dorsal spinal gray matter at a depth of approximately 1 mm. Traces demonstrate an increase in diaphragm EMG, arterial pressure and inspiratory pressure during the period of triggered stimulation, with effects persisting up to several minutes following termination of stimulation. B) Average left and right diaphragm EMG burst amplitude post-intraspinal stimulation, illustrating a robust increase in left (ipsilateral to stimulating electrode) diaphragm EMG amplitude. Modest enhancement of diaphragm EMG activity was also observed contralateral to stimulation. C) Average mean arterial pressure (MAP) and heart rate (HR) during

and following intraspinal stimulation. Robust increases in physiological parameters were observed following stimulation. D) Overlay of tracheal pressure waveforms before stimulation (black) and during stimulation (red) and average tracheal pressure defection before, during and after stimulation indicate an increase in the occurrence of negative pressure deflections just prior to inspiration.

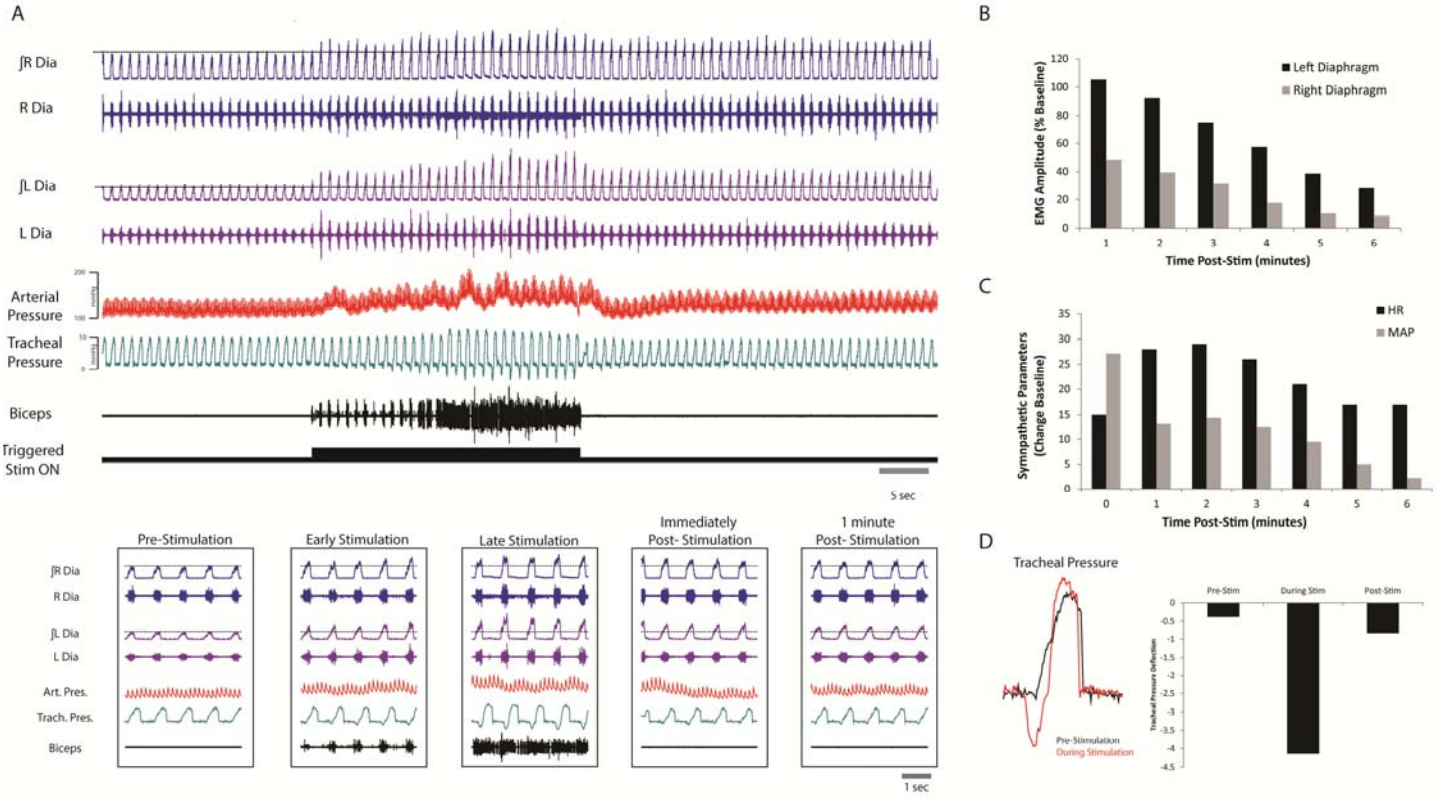

Figure 8. Mid-cervical intraspinal microstimulation using the NeuroChip microprocessor augments respiratory and sympathetic output. A) Compressed (top) and expanded (bottom) traces of left and right diaphragm EMG, arterial blood pressure, tracheal pressure, and bicep activity before, during, and following hypoglossal triggered intraspinal (C4) stimulation (400µA; <100Hz).

Thus, we are poised for success in year 2 of the grant, and are planning larger scale NeuroChip studies as described elsewhere in this progress report.

University of Washington (July 2015-present)

Since establishing the sub-contract at the University of Washington in July, the following activities have been accomplished.

- **Visit to the University of Florida (Major task 6, subtask 1)**: UW Research Scientist Michael Sunshine traveled to the University of Florida for 3 days in August. He trained on the C2 hemisection model from experts at UF, and participated in a number of closed-loop stimulation experiments. He also traveled with the Neurochip circuit, setting this up in the lab at UF and validating data collection on the respiratory model system. In addition, Mr. Sunshine began to train the UF team to implant chronic intraspinal stimulation (ISMS) electrode arrays needed for long-term treatment studies. These are the first steps to enable the UF team to use the Neurochip and ISMS arrays for chronic treatment studies as described in Major Task 1, subtask 1. This will enable replication of experiments between the two sites. A follow-up visit will occur in the first quarter of 2016 (likely February 10-11).

- **Prepare for respiratory studies (Major Task 4):** In the 4 months since the beginning of the sub-contract, all equipment has been procured and set up to perform respiratory experiments at UW. This includes purchasing and setting up respiratory pressure monitors, plethysmography chambers, amplifiers and CED recording system. This is a major accomplishment as the

respiratory system is a new model organ system for the UW team. Thus all sub-tasks of Major task 4 are now completed on schedule.

- **Establish and validate model of C2 hemisection (Hx):** After training on this new injury model at the University of Florida, we have implemented this injury reliably at the University of Washington. This adds to our repertoire of cervical injuries performed in the lab, most commonly the contusion injury that will be explored in the second phase of this project. This is key step in preparing for Major Task 5 subtask #3 to be completed next.

- **Perform closed-loop intraspinal microstimulation (ISMS – Major Tasks 5 and 7)**: We have performed acute stimulation of the phrenic motor pool in both intact animals, as well as after acute C2Hx. We quantified the stimulation amplitude required to produce ipsilateral activation of the diaphragm using both rack-mounted (TDT) and Neurochip (NC) hardware. When stimulation is in-phase with respiratory rhythm in a spinal-intact animal, additional stimulation evoked activation is limited. Following C2Hx, however, the diaphragm is robustly excited by closed-loop ISMS delivered using both TDT and Neurochip circuits (Figure 9). This sets the stage for closed-loop treatment studies of Major Tasks 5 and 7 to begin on schedule at both UW and UF in the coming months.

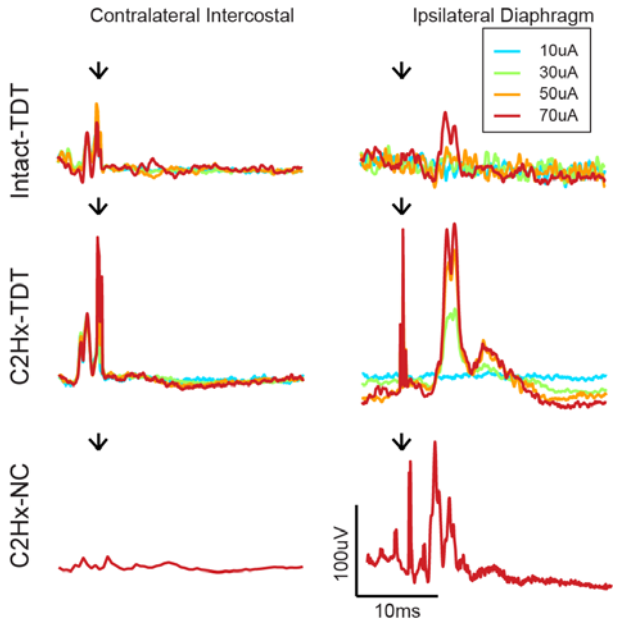

Figure 9: Response to closed loop stimulation of the respiratory circuit. Stimulation is triggered by activity of the contralateral intercostal muscle (left column). Stimulation is delivered to the ipsilateral/ipsilesional diaphragm via intraspinal microstimulation (ISMS) of the phrenic motor circuit. ISMS was delivered at C4 via an array of electrodes located 1 mm below the dorsal. Stimulation timing was determined via recording of the contralesional intercostal EMG. Traces illustrate stimulus triggered averages of EMG aligned on the stimulation delivered at black arrow. The diaphragm response is much greater following C2 hemisection (C2HX) injury compared to the pre-injury condition (top row). The diaphragm response was robust and scaled with stimulus amplitude (colored lines) following C2Hx. This closed-loop stimulation was repeated at the highest current using the self-contained Neurochip (NC) circuit (bottom row. This paradigm will be used to deliver chronic, activity-dependent stimulation in long-term treatment studies during year 2.

Summary

Our initial findings have fully supported the central hypothesis of this project that closed-loop activation of the phrenic nucleus is feasible before and after a C2Hx. In addition, this result has been reproduced at two sites using complementary approaches. Interestingly, we also have indications of persistent effects (i.e. beyond stimulation intervals) that appear early suggesting ISMS may have profound neuroplastic influences in the phrenic motor system.

3A. Opportunities for Training and Professional Development

Major Task 1 involved a close interaction between Drs. David Baekey and Kristi Streeter (Post-Doctoral Fellow) through which Dr. Streeter gained additional experience in respiratory neurophysiology and multi-electrode array recordings (Dr. Baekey's expertise).

Major Task 2 is being conducted by Ms. Lynne Mercier, a fourth year graduate student, who worked under the guidance of Drs. Baekey and Fuller and gained additional neurophysiology experience.

Mr. Michael Sunshine (Univ. Washington) visited the Gainesville campus and learned new surgical procedures and has been interacting closely on experimental design with Drs. Fuller and Moritz. He is now anticipating going to graduate school.

3B. Dissemination of Results

Nothing to Report

3C. Plans for Accomplishments of Goals

Given that we are only into the first half of Year-01 official funding, our plan is to complete the above studies and submit findings for publication. In addition, we want to move ahead and capitalize on the main focus of the project – namely, closed-loop ISMS – which the above data nicely supports even so early on in the project. The fundamental hypothesis to be tested, initially at the University of Washington, is that Neurochip-driven ISMS can evoke an increase in inspiratory tidal volume during the period of stimulation (i.e., closed loop spinal stimulation increases tidal volume). In addition, we propose that temporally matching the threshold trigger with the recorded respiratory EMG will more effectively evoke plasticity (manifest as a persistent increase in tidal volume following stimulation) 2. "ramping" the patterns if spinal stimulation to match the typical "phrenic ramp" during inspiration will more effectively modulate tidal volume. This approach is to "fine tune" ISMS and is consistent with our SOW milestones. UF will initiate ordering Neurochips so that we can reproduce UW results per the grant. UF will need to send 1-2 persons to UW for training

3D. Literature Cited

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4. Impact

Too early into the project to be able to define impact.

5. Changes/Problems

No significant problems or departures from the

6. Products

Nothing to report at this early stage of investigation. A draft of a manuscript describing the closed-loop proof-of-concept study (Major Task 2) has been prepared.

7. PARTICIPANTS AND OTHER COLLABORATIVE ORGANIZATIONS:

A. Individuals Working on Project

Name	Project Role	Nearest Person Month Worked Equivalents	Contribution to Project
University of Florida			
Paul J. Reier, Ph.D.	Project Director	0.6	Oversees entire project; conducts experiments related to Major Task 3
David D. Fuller, Ph.D.	Co-I	0.24	Functions as Associate Project Director

			responsible for overseeing neurophysiology studies and interfacing with Seattle researchers
David Baekey, Ph.D.	Co-I	0.24	Multi-electrode array recordings for Major Task 1
Kristi Streeter, Ph.D.	Post-Doctoral Fellow	12.0	Conducted recordings for Major Task 1
Elisa Gonzalez-Rothi, Ph.D.[1]	Post-Doctoral Fellow	5.0	Epidural stimulation studies for Major Task 2.
Lynne Mercier[2]	Graduate Student	9.0	Conducted ISMS proof-of-concept study for Major Task 2.
Lucy Denholtz	Technician	3.0	Assists with surgeries and histological procedures.
Savannah Posgai[3]	Technician	6.0	Assists with surgeries and histological procedures.
University of Washington			
Chet Moritz, Ph.D.	Sub-contract project director	1.73	Oversees project Major Tasks to be done at U. Wash.
Michael Sunshine	Graduate Student	12.0	Primary person responsible for carrying out U. Washington studies. Mr. Sunshine is an extremely talented and mature investigator with outstanding expertise.

[1]Dr. Elisa Gonzalez-Rothi's effort was supported via internal resources.
[2]Ms. Lynne Mercier was supported via a NIH Training Grant in the Department of Physical Therapy
[3]Ms. Savannah Posgai's effort was supported via internal resources

B. Active and Other Support Changes
Nothing to report

C. Other Organizations Involved as Partners.

Organizations Name: University of Washington (subcontract)
Partner's Contribution to the Project: Expertise in ISMS and provision of required neural interface.
Personnel exchanges.
Replication studies.

8. SPECIAL REPORTING REQUIREMENTS:

See Quad Chart next page.

Plasticity and Activation of Spared Intraspinal Respiratory Circuits Following Spinal Cord Injury

W81XWH-14-1-0625 (UNCLASSIFIED)
SC120209

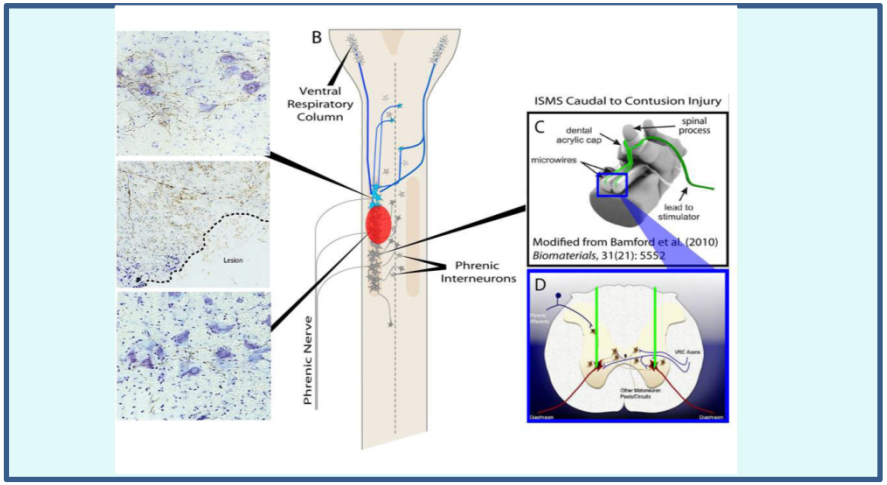
PI: Paul J. Reier, Ph.D. **Org:** University of Florida Coll. Med. and McKnight Brain Institute **Award Amount:** $494,778

Study/Product Aim(s)

- **Aim 1:** To determine whether physiologically-based ISMS (intraspinal microstimulation) below a C3/4 lateralized contusion or C2 hemisection will enhance the respiratory activity of PhMNs and the diaphragm.
- **Aim 2:** To demonstrate with neuroanatomical and electrophysiological methods that patterned electrical stimulation of spinal circuitry, caudal to the site of injury, will promote altered connectivity in conjunction with changes in PhMN and diaphragm function.

Approach

This project is to assess the effects of epidural stimulation vs. ISMS on phrenic motoneuron/diaphragm function and phrenic circuit connectivity following cervical spinal cord injury. Terminal electrophysiological experiments will first be performed to map the phrenic motor circuit in rats 1-2 months post C3/4 contusions using microarray assemblies. Terminal electrophysiological comparisons will then be obtained of epidural and ISMS stimulation on activation of the phrenic motor circuit after chronic C2 hemisection (C2Hx; one month post-SCI). The studies will then move to in vivo tests following C2Hx and C3/4 contusions. ISMS will entail open- and closed-loop stimulation.



Illustrated here is the overall goal of the project testing spinal stimulation on recovery of diaphragm function following interruption of descending respiratory drive pathways to the phrenic nucleus.

Timeline and Cost

Activities	CY 15	16		
Administrative Task 1				
Major Tasks 1-5				
Major Tasks 6-11				
Estimated Budget ($K)	**$200***	**$200***		

* Direct Cost

Updated: 10/28/2015

Goals/Milestones:

CY15 Goal – All Major Tasks in Progress

- ☐ Major Tasks 1-2: To map phrenic circuit discharge patterns following a C3/4 lateralized contusion injury and terminal electrophysiological comparison of epidural stimulation and ISMS after chronic C2 hemisection
- ☐ Major Task 3: Analysis of Tissue Responses to Microwire Implantation
- ☐ Major Task 4: Prepare for respiratory studies
- ☐ Major Task 5: Phase I of closed-loop intraspinal stimulation of phrenic motor neurons (PhMNs) after C2Hx.

CY16 Goal –

- ☐ Major Tasks 6-11: Open- and closed loop studies in spinal-contused rats. Replication of most promising stimulation approach with follow up neuroanatomical studies.

Comments/Challenges/Issues/Concerns

- This project has met with considerable delay related to acquisition of ACURO approval. Only 5-6 mos. of funded research reported. **No cost extension anticipated.**

Budget Expenditure to Date (10-15-2015) including Sub-Contract:
$ 73,303.57